A systematic approach for extraction of phenolic compounds using parsley (*Petroselinum crispum*) flakes as a model substrate[†]

Devanand L Luthria,* Sudarsan Mukhopadhyay and Albert L Kwansa

Food Composition Laboratory, Beltsville Human Nutrition Research Center, ARS, USDA, Beltsville, MD 20705-3000, USA

Abstract: The impact of extraction methodology and polarity of extraction solvents on the assay of phenolic compounds was investigated using parsley (*Petroselinum crispum*) flakes as a model substrate. This systematic study was undertaken to address substantial variations in the extraction procedures, solvents and conditions as described in the recent literature. Five different extraction procedures [shaking, vortex mixing, sonication, stirring and pressurized liquid extraction (PLE)] and three different solvents (methanol, ethanol and acetone), with five different solvent to water ratios per solvent, were used for extraction. Extracts were analyzed for phenolic content by high-performance liquid chromatography and Folin–Ciocalteu assays. The yields of phenolic compounds extracted with a pressurized liquid extractor were comparable to or better than those of four classical extraction procedures. Optimum extraction efficiency with PLE was obtained when extractions were performed with four extraction cycles using ethanol–water (50:50, v/v). The amount of apiin (4,5,7-trihydroxyflavone 7-apiosylglucoside) and malonylapiin (apigenin malonylapiosylglucoside) isolated from parsley varied with the composition of extraction solvent. Apiin extractability was found to be a maximum when the solvent (ethanol, methanol or acetone) to water ratio was 30:70 (v/v), whereas higher amounts of malonylapiin were isolated with a reverse solvent to water ratio (70:30, v/v). Malonylapiin was not detected when parsley samples were extracted with organic solvent to water ratios of 10:90 (v/v) and 30:70 (v/v).
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INTRODUCTION

Phenolics are one of the most diverse groups of phytochemicals that are universally distributed in fruits, vegetables and herbs. These compounds possess at least one phenol moiety (an aromatic ring with one or more hydroxyl substituents) and approximately 8000 phenolic compounds have been isolated from natural resources.[1] Epidemiological, clinical and laboratory studies suggest that consumption of fruits, vegetables and herbs may reduce the risk of chronic diseases such as coronary heart disease and cancer.[2,3] The beneficial effect of phenolic compounds has partially been ascribed to the antioxidant activity of the phenolics.[4,5] Hence accurate estimation of the levels of the phenolic compounds in plants is critical but is limited by problems of analysis.[6] Structural diversity and solubility impose a significant challenge in the extraction and analysis of phenolics.[7] Reports estimate that approximately 30% of the errors in analytical measurements come from the sample preparation step.[8]

The first step in any analysis is extraction of the analyte from the source material. Extraction of phenolic compounds from plant material is influenced by various parameters such as solvent polarity, particle size, extraction procedures and conditions. The impact of the extraction of phenolic compounds on the analysis has often been overlooked as substantial variations in the extraction procedures and solvents are documented in the recent literature.[6,7] Several solvents such as methanol, ethanol, acetone, water, ethyl acetate and, to a lesser extent, propanol, dimethylformamide, dimethyl sulfoxide and their combinations have been used for the extraction of different classes of phenolic compounds.[6–13] The reasoning for the selection of a particular extraction solvent or solvent mixture in addition to the extraction procedure is frequently not well studied and/or not clearly documented.

There have been two systematic studies on the comparison of extraction procedures in the assay of phenolics.[14,15] Alonso-Salces *et al*. studied the

optimization of pressurized liquid extraction (PLE) for the determination of polyphenols in apple with varying composition of aqueous methanol.[14] Torti *et al.* compared shaking, stirring and homogenization procedures for the extraction of phenolics from fresh leaves of two species of plants that differ in leaf toughness (*Acomastylis rossii* and *Ouratea lucens*).[15]

The objective of this work was to develop a systematic approach for the extraction of phenolic compounds from a food matrix. Parsley (*Petroselinum crispum*) was selected as a model food substrate as it one of the most common herbs consumed globally and is one of the richest sources of a commonly occurring phenolic aglycone, apigenin. In parsley, apigenin is usually found in conjugate form as apiin or its analogs.[16,17] To achieve our objective, we compared the extraction efficiency of PLE with four commonly used extraction techniques (shaking, stirring, vortex mixing and sonication) using MeOH–H_2O (62.5:37.5, v/v) as described by Justesen.[16] In addition, we studied the influence of solvent polarity on the extraction efficiency of phenolic compounds from parsley with PLE using three different solvents EtOH, MeOH and acetone, with five different solvent to water ratios. Finally, the impact of the number of extraction cycles on the extraction efficiency of phenolics was also evaluated. Phenolics were assayed by two independent analytical procedures namely Folin–Ciocalteu (FC) and high-performance liquid chromatographic (HPLC) assays.

MATERIALS AND METHODS
Plant material
Dried parsley (*Petroselinum crispum*) flakes were purchased from a local grocery store (Giant, Beltsville, MD, USA). Immediately, after receipt, the material was subdivided, flushed with nitrogen and stored at $<-60\,°C$ until analysis.

Chemicals
HPLC-grade solvents, methanol and acetonitrile were purchased from Fisher Chemicals (Fair Lawn, NJ, USA) and HPLC-grade acetone from Burdick & Jackson (Muskegon, MI, USA). Denatured anhydrous ethanol was obtained from Mallinckrodt (Paris, KY, USA). HPLC-grade formic acid was procured from Aldrich Chemical (Milwaukee, WI, USA) and apiin standard from Chromadex, (Santa Ana, CA, USA). Diatomaceous earth (Celite 545) and Ottawa sand were purchased from Fisher Chemicals. FC reagent, gallic acid and sodium carbonate for the assay of total phenolics (TP) were obtained from Sigma Chemical (St Louis, MO, USA). Deionized water (18 Ω) was prepared using a Millipore Milli-Q purification system (Millipore, New Bedford, MA, USA). Poly(vinylidene difluoride) (PVDF) syringe filters with pore size 0.45 μm were purchased from National Scientific (Duluth, GA, USA).

Comparison of extraction procedures
Dried parsley flakes were ground with a coffee grinder and passed through a standard sieve No. 20 (particle size <825 μm). The dried powder was extracted with MeOH–H_2O (62.5:37.5, v/v). Extractions were carried out using five different procedures namely, shaking, vortex mixing, stirring, sonication and PLE.

Extractions were carried out using the same solid to solvent ratio and solvent mixture as described by Justesen.[16] For each extraction, approximately 250 ± 1 mg of powdered parsley were placed in a 16×125 mm screw-capped vial and 10 mL of MeOH–H_2O (62.5:37.5, v/v) solvent mixture were added. The vial was then placed on a Model 75 Wrist Shaker (Burrell, Pittsburgh, PA, USA) at a high speed (setting No. 10) for 30 min. For sonication, vials were placed in a sonicator bath (Model 2510, Branson Ultrasonic, Danbury, CT, USA) at $40\,°C$ for 30 min. Stirring was carried out by adding one 8×1.5 mm PVDF-coated magnetic bar to each vial and placing the vials in a 100-mL beaker on a Model PC 351 hot-plate/stirrer (Corning, Corning, NY, USA) at ambient temperature. Extraction with vortex mixing was performed by vortex mixing the vials for 2 min (three times) on a Daigger Vortex Genie 2 (Scientific Industries, Bohemia, NY, USA). After extraction with the different procedures, the mixture was centrifuged at a low speed ($10\,000 \times g$) for approximately 10 min. The supernatant was transferred into a 25-mL volumetric flask. The residue was resuspended in an additional 5 mL of MeOH–H_2O (62.5:37.5, v/v), gently mixed manually for 30 s and centrifuged for 5 min. The supernatant was combined with the first extract. The volume of combined supernatant was made up to 25 mL with extraction solvent and 2-mL aliquots of extracts were filtered through a 0.45-μm PVDF syringe filter for phenolic assay by the FC method and HPLC analysis. For each sample, extraction and analyses were carried out in triplicate.

A Model ASE 200 Accelerated Solvent Extractor (ASE) (Dionex, Sunnyvale, CA, USA) was used for PLE. Aliquots of 250 ± 1 mg of dried powdered parsley flakes were placed in an 11-mL stainless-steel extraction cell. Two circular cellulose filters (size 1.983 mm, Dionex) were placed at the bottom of the extraction cell in order to prevent suspended particles from entering the collection vials. The remaining void volume in the cell was filled with Ottawa Sand. Both extraction cells and collection vials were arranged appropriately in the two designated carousels. Extractions were carried out with MeOH–H_2O (65:35, v/v) solvent mixture. Extractions were performed at 1000 psi, with a 5-min equilibration time, a 10-min static time and a 90-s purge time for each extraction cycle. Four extraction cycles were performed for each sample. Only for comparison with the other extraction procedures, the static time for PLE was set to 5 min in order to complete the extraction per sample within 30 min. The extractions were carried out at $40\,°C$ and a total of about 15 mL of solvent was obtained for

the four extraction cycles with the flush volume set at 75%. The extracts were collected in 60-mL amber-colored sample vials fitted with Teflon-coated rubber caps (I-CHEM, New Castle, DE, USA). Each extract was transferred to a 25-mL volumetric flask and the total volume was adjusted to 25 mL with the appropriate solvent mixture. Aliquots of parsley extracts were filtered through a 0.45-μm PVDF syringe filter prior to analysis of phenolics by FC and HPLC assays. Triplicate extractions, FC assays and HPLC analyses were carried out for each sample.

Comparison of solvent composition

A systematic variation of different proportions of three solvent mixtures (90:10, 70:30, 50:50, 30:70 and 10:90, v/v) of EtOH, MeOH and acetone to H_2O, was used to compare extraction efficiency of phenolic compounds from parsley flakes. The volume of combined extract was adjusted to 25 mL with the corresponding extraction solvent and appropriate aliquots were filtered through a 0.45-μm PVDF syringe filter prior to TP and HPLC assay. Triplicate extractions, FC assays and HPLC analyses were carried out for each sample. All extractions were carried out with approximately 250 ± 1 mg of dried powdered parsley flakes (particle size <825 μm) by using ASE and under the same conditions as described in PLE section.

Investigation of number of extraction cycles for PLE

In these experiments, the extracts from the four extraction cycles were flushed into four different collection vials. The volume of each extract was adjusted to 25 mL and appropriate aliquots were filtered through a 0.45-μm PVDF syringe filter prior to assay for phenolics by FC and HPLC analysis.

Determination of total phenolics (TP) by FC assay

The TP content was determined using the FC assay with gallic acid as a standard on a Lambda 25 spectrophotometer (Perkin-Elmer, Wellesley, MA, USA).[18] FC assay was carried out by pipetting 60 μL of parsley extract into an 8-mL amber-colored vial. This was followed by addition of 4.74 mL of water. This mixture was vortex mixed for $10-20$ s and 300 μL of FC reagent were added. The mixture was vortex mixed for an additional $20-30$ s and 900 μL of filtered $200 \, g \, L^{-1}$ sodium carbonate solution were added after 1 min and before 8 min of addition of the FC reagent. This was recorded as time zero; the mixture was vortex mixed for $20-30$ s after addition of sodium carbonate. After $2 \, h \pm 3$ min, at ambient temperature, the absorbance of the colored reaction product was measured at 765 nm. A calibration curve was created using standard gallic acid solutions each time an analysis was run. The level of TP in the extract was calculated from the calibration curve. Results were expressed in mg of gallic acid equivalent per gram (mgGAE g^{-1}) of dried parsley flakes.

Influence of solvent composition on extraction efficiency of standard apiin

To evaluate the influence of solvent composition on extraction efficiency, 5.5 mg of apiin standard was dissolved in 1.5 mL of EtOH$-H_2O$ (2:1, v/v). For the recovery experiment, 150 μL of this mixture were added to powdered parsley flakes (100 mg). Samples were extracted by PLE using 5-mL extraction cells with a flush volume setting of 10% at 40 °C. All other PLE extraction conditions were the same as previously described. Extractions were carried out in triplicate with two solvent mixtures containing different EtOH to H_2O ratios (30:70 and 70:30, v/v). In a control experiment, 150 μL of solvent mixture (EtOH$-H_2O$, 2:1, v/v) without apiin standard were added to parsley flakes prior to extraction. Triplicate analyses were carried out for each set.

Determination of phenolic compounds by HPLC

Samples (20 μL of extract) were separated using an HPLC system (Model 1050 pump, Hewlett-Packard, Palo Alto, CA, USA) coupled with a photodiode-array detector (DAD) (Series 1040M, Series II) and an autosampler (Series 1050) operated by HP Chem-Station software. A reversed-phase C_{18} Luna column (Phenomenex, Torrance, CA, USA; 150×4.6 mm i.d., particle size 5 μm), preceded by a guard column (Phenomenex, 4×3.0 mm i.d.) with the same stationary phase was used for HPLC and liquid chromatographic$-$mass spectrometric (LC$-$MS) analysis. The column and the guard column were thermostatically controlled at 40 °C and the flow-rate was set to $0.5 \, mL \, min^{-1}$. The mobile phase consisted of two solvents: acetonitrile (A) and water$-$formic acid (99.9: 0.1, v/v) (B). The solvent gradient in volumetric ratios of solvents A and B was as follows: from 0 to 30 min, 10 to 30% A; from 30 to 50 min, 30 to 60% A; from 50 to 55 min, 60 to 100% A; from 65 to 70 min, 100 to 10% A; and from 70 to 75 min, 10% A. Dual wavelengths (270 and 350 nm) were used to detect the eluent composition. Detection was carried out using a photodiode-array UV detector. As an authentic purified standard of malonylapiin was not available from commercial sources, comparison of extraction efficiency was calculated on a percentage basis from the HPLC peak areas of identified phenolic compounds. Stock standard solutions ($40 \, mg \, L^{-1}$) of apigenin and apigenin-7-O-glucoside (Extrasynthèse, Genay, France) were used as reference standards to monitor the performance of HPLC on a regular basis.

Identification of phenolic compounds by LC$-$MS

An Agilent 1100 LC system coupled with a diode-array and MSD (SL) detectors (Agilent, Palo Alto, CA, USA) was used to identify individual phenols. For LC$-$MS analysis, the same column, flow-rates and gradients were used as described for HPLC. Mass spectra were acquired in the positive ion mode at both low and high fragmenter voltages (70 and 250 V). The instrument was set to scan from 100 to 2000 mass

units. The temperature of the drying gas was 350 °C at a flow-rate of 13 L min^{-1} and a nebulizer pressure of 50 psi. The LC system was directly connected to the mass spectrometer with no stream splitting. Identification of phenolic compounds was achieved by comparison of LC–MS data with those reported in the literature.

Statistical analysis

All statistical analyses were conducted using PROC MIXED in SAS® v. 9.1.2 (SAS Institute, Cary, NC, USA). Heterogeneous replicate variability among methods and solvents mixtures was modeled by specifying variance groups using the GROUP = option of the REPEATED statement. For each extraction procedure, a one-way ANOVA was conducted with subsequent means comparisons (using the PDMIX800 freeware SAS macro) to identify differences among extraction methods.

RESULTS AND DISCUSSION

The levels of phenolic compounds in plants, fruits, vegetables and herbs are influenced by genotype, agronomic practices, maturity at harvest and post-harvest storage, climatic, regional and processing conditions.[19–23] To eliminate these variations, all analyses were performed with a homogeneous sample obtained by grinding 250 g of dried parsley flakes procured from a single source. The ground sample was passed through a standard sieve (size 20) and the fraction with particle size <0.850 mm was collected, mixed and stored in a refrigerator in dark amber-colored bottle.

Identification of phenolic compounds

Figure 1 shows three HPLC traces for the parsley flakes extracted with three different EtOH to H$_2$O ratios: (A) 30:70, (B) 50:50 and (C) 70:30 (v/v). The tentative structural assignments of eight peaks are given in Table 1. Structural elucidations were done by UV and LC–MS analysis, and also by comparing these data with reported literature data.[16,24,25] Significant variations in the HPLC profiles of the phenolic compounds were observed as the extraction solvent compositions were changed. The HPLC profiles of parsley samples extracted with EtOH–H$_2$O (30:70, v/v) [Fig. 1(A)] showed one major peak (peak 1, 78% of summed HPLC peak area of identified phenolic compounds) that was identified as apiin. The combined area of the other four minor peaks was less than 20%. In comparison, the HPLC profile of the same parsley samples extracted with EtOH–H$_2$O (50:50 and 30:70, v/v) [Fig. 1(B) and 1(C)] contained two major compounds that were identified as apiin (peak 1) and malonylapiin (peak 4). In Fig. 1(B), apiin and malonylapiin make up 40 and 42%, respectively,
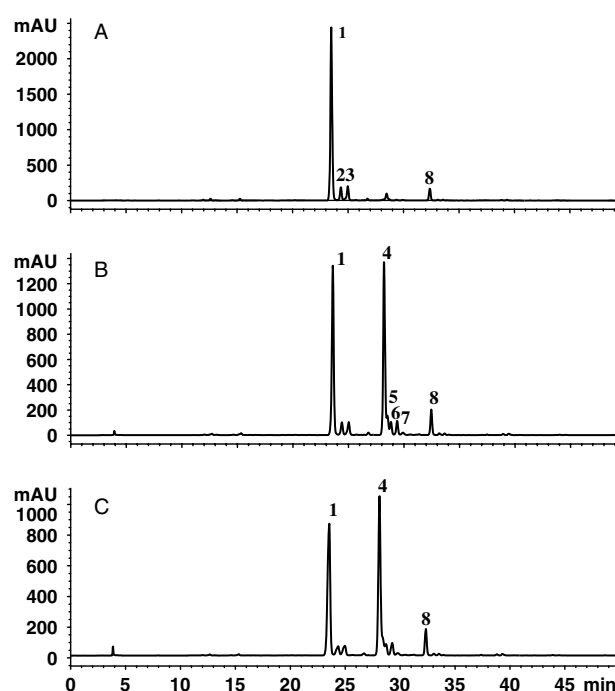

Figure 1. HPLC profile of dried parsley flakes extracted with different solvent mixtures by PLE. (A) EtOH–H$_2$O (70:30, v/v); (B) EtOH–H$_2$O (50:50, v/v); (C) EtOH–H$_2$O (30:70, v/v). The peaks were identified as (1) apigenin-7-apiosylglucoside (apiin), (2) diosmetin apiosylglucoside, (3) diosmetin apiosylglucoside isomer, (4) apigenin malonylapiosylglucoside (malonylapiin), (5) apigenin malonylapiosylglucoside (malonylapiin), (6) diosmetin malonylapiosylglucoside, (7) apigenin malonylglucoside and (8) apigenin acetylapiosylglucoside (acetylapiin).

Table 1. Peak assignment for the phenolic compounds extracted with EtOH–H$_2$O (70:30, v/v) from dried parsley flakes

Peak No.	Retention time, t_R (min)	MS data: m/z		UV data: λ_{max} (nm)	Structural identification	References
		ES +ve: $[M + H]^+$ product ion	ES −ve: $[M - H]^+$ fragment aglycone ion			
1	23.4	565	271	266, 338	Apigenin-7-apiosylglucoside (apiin)	16,24
2	24.2	595	301	253, 266, 346	Diosmetin apiosylglucoside	16,24
3	24.8	595	301	253, 266, 346	Isomer of diosmetin-apiosylglucoside	16,24
4	27.9	651	271	266, 336	Apigenin malonylapiosylglucoside (malonylapiin)	16,24,25
5	28.3	651	271	266, 336	Apigenin malonylapiosylglucoside (malonylapiin)	16,24,25
6	28.8	681	301	253, 266, 346	Diosmetin malonylapiosylglucoside	16,24
7	29.1	519	271	253, 266, 346	Apigenin malonylglucoside	16,24,25
8	32.0	607	271	264, 336	Apigenin acetylapiosylglucoside (acetylapiin)	16,24

of the summed HPLC peak areas of all identified phenolic compounds. In Fig. 1(C), these values were 39 and 43%.

Extraction procedures

Dried and ground parsley flakes were extracted with the same solvent mixture, MeOH–H_2O (62.5:37.5, v/v), and the same solid to solvent ratio (10 mL solvent for 250 mg of sample) as described by Justesen.[16] As details of the extraction procedure were not described, samples were extracted with four commonly employed extraction procedures (shaking, sonication, stirring and vortex mixing). Extractions with PLE were performed to compare the extraction efficiency of PLE with those of four commonly used procedures. The MeOH to H_2O ratio was slightly modified for PLE [65% instead of 62.5% (v/v) MeOH] owing to the limitation of the ASE instrumentation, which allowed only increments of 5% (by volume) for each solvent. As the total volume of solvent used by PLE for all four cycles per extraction was 15 mL, an additional 5-mL gentle wash was added to the procedure described by Justesen to compare extraction efficiencies using the same solvent volume. The extraction time for each extraction was approximately 30 min for all five procedures. However, the extraction workup time with PLE was significantly reduced owing to the elimination of decantation and centrifugation steps. In addition, exposure to sample and solvent vapor was also reduced with PLE owing to automation of the extraction, decantation and nitrogen purging steps.

The TP content of all extracts was estimated by two independent assays (FC assay and HPLC peak area). Figure 2 indicates similar trends in the extraction yield of phenolic compounds measured by two assays. Both assays indicated that PLE provided a marginal

increase in extraction yields of TP as compared with the other four extraction procedures (shaking, sonication, stirring and vortex mixing). This can be attributed to the differences in extraction principle, as PLE extractions were carried out at 40 °C under 1000 psi in an inert nitrogen atmosphere. The shaking extraction protocol provided the lowest extraction yield as measured by FC assay (79% of the yield obtained by PLE) and HPLC analysis (79% of the yield obtained by PLE). The yields obtained with the other three extraction procedures ranged from 81 to 92% of that of the PLE procedure. A one-way ANOVA test of the HPLC and FC data showed some statistical differences in the phenolic content among the five extraction methodologies assayed by two independent procedures (Table 2).

Extraction solvents

The structural diversity of phenolic compounds presents a significant challenge for developing a uniform methodology that is suitable for extraction of all phenolics or a specific class of phenolic compounds. The issue of developing a satisfactory extraction procedure is further complicated as phenolics are not uniformly distributed in plants at the tissue, cellular and subcellular levels. In addition, these compounds can be founds in free, conjugated and polymeric forms or may coexist as complexes with carbohydrate, protein or other plant components. All of the above factors directly impact the solubility of phenolics in different solvents. In spite of all the above issues, sample extraction has often been overlooked and most literature citations are either not well documented or poorly optimized. To evaluate the impact of solvent polarity on the solubility of phenolics, a systematic extraction approach using three commonly used solvents (EtOH, MeOH and acetone) and five different solvent to water ratios (90:10, 70:30, 50:50, 30:70 and 10:90, v/v) was performed. PLE was used to evaluate the extraction efficiency of phenolic compounds based on the performance described in the previous section. Approximately 250 ± 1 mg of powdered parsley flakes were extracted in triplicate with 15 different solvent mixtures by the PLE procedure.

The efficiency of extraction of apiin and malonylapiin from parsley flakes was calculated by dividing the HPLC peak area of apiin and malonylapiin extracted with each solvent mixture by the that of apiin and malonylapiin extracted with EtOH–H_2O (30:70, v/v) and EtOH–H_2O (70:30, v/v) respectively, as the maximum amount of apiin was extracted with EtOH–H_2O (30:70, v/v) whereas the maximum amount of malonylapiin was extracted with EtOH–H_2O (70:30, v/v). The extraction efficiency with MeOH–H_2O and acetone–H_2O solvent mixtures was calculated in a similar manner.

The amount of apiin and malonylapiin extracted from parsley flakes varied with extraction solvent composition. The maximum amount of malonylapiin
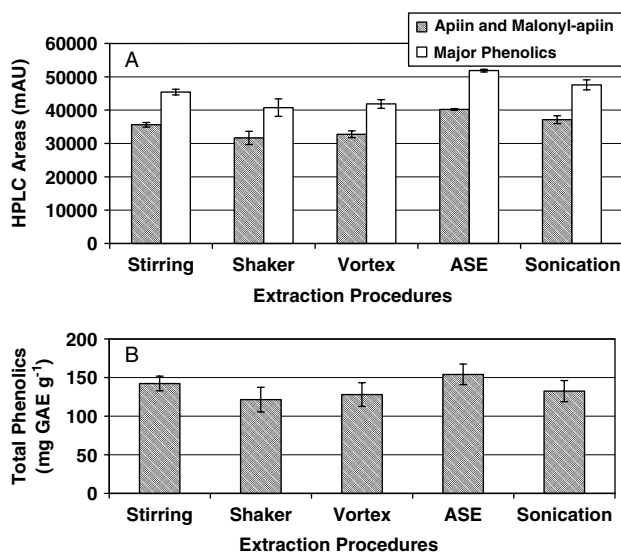

Figure 2. Comparison of extraction efficiency of phenolic compounds with MeOH–H_2O by five popular extraction procedures using two different assays. (A) HPLC analyses with diode-array detection (350 nm) with sum of areas under chromatographic peaks of apiin and malonylapiin and all identified major phenolics compounds. (B) Total phenolics by Folin–Ciocalteu assay.

Table 2. One-way ANOVA test of the phenolic content extracted using five extraction procedures assayed by two independent analytical procedures: (A) Folin–Ciocalteu assay and (B) HPLC analysis (sum of peak areas of apiin and malonylapiin)

(A)

Extraction method	Total phenolics (mgGAE g^{-1})	Standard error	Error degrees of freedom	t-Value	Pr > |t|	Letter group[a]
PLE	15.5	0.2	20	70.5	<0.0001	A
Stirring	14.2	0.4	20	33.5	<0.0001	B
Sonication	13.4	0.2	20	61.2	<0.0001	B, C
Vortex mixing	12.8	0.2	20	58.6	<0.0001	C
Shaking	12.1	0.4	20	4.0	<0.0165	C

(B)

Extraction method	HPLC area (apiin + malonylapiin)	Standard error	Error degrees of freedom	t-Value	Pr > |t|	Letter group[a]
PLE	40 192	132.1	12	304.2	<0.0001	A
Stirring	37 138	562.8	12	66.0	<0.0001	B
Sonication	35 599	562.8	12	63.3	<0.0001	B
Vortex mixing	32 761	562.8	12	58.2	<0.0001	C
Shaking	31 651	1151.4	12	27.5	<0.0013	C

[a] Estimation of phenolics associated with different letters are significantly different at $\alpha \leq 0.05$.
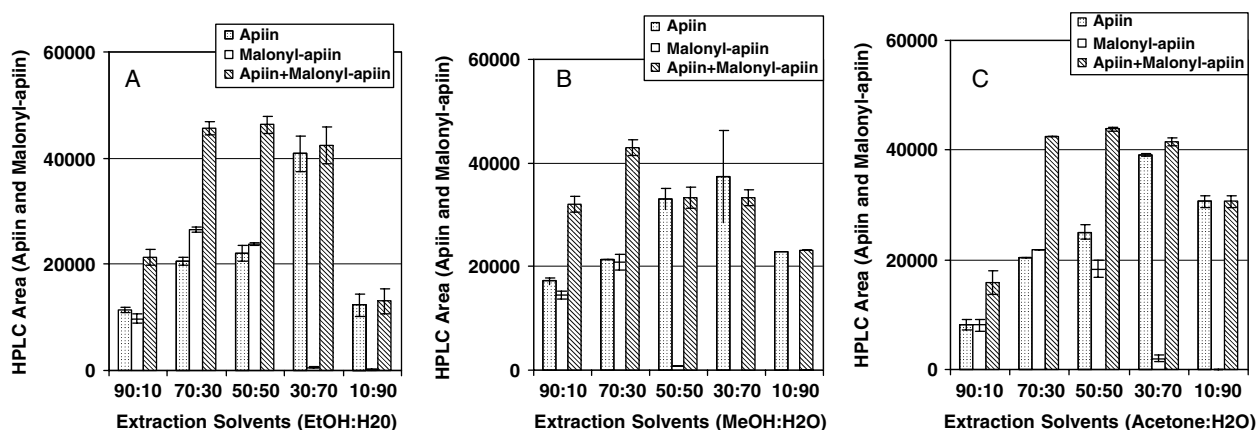
Figure 3. Distribution pattern of major phenolic compounds (apiin and its two malonic ester isomers) extracted from dried parsley flakes with 15 different aqueous solvent mixtures (solvent:H$_2$O 90:10, 70:30, 50:50, 30:70, 10:90, v/v) of varying polarity. (A) EtOH–H$_2$O; (B) MeOH–H$_2$O; (C) acetone–H$_2$O.

($t_R \approx 28.2$ min) was extracted with 70% EtOH. However, intermediate yields (90 and 37%) of malonylapiin were extracted with 50 and 90% EtOH. The yields of malonylapiin were <3% when extracted 30% and 10% EtOH [Fig. 3(A)]. The optimum yield of apiin was obtained with EtOH–H$_2$O (30:70, v/v). The yields of apiin extracted with other EtOH–H$_2$O solvent mixtures (90:10, 30:70, 50:50 and 10:90, v/v) varied between 28 and 54% [Fig. 3(A)]. The combined yields of apiin and malonylapiin were comparable with three different EtOH–H$_2$O solvent mixtures (30:70, 50:50 and 70:30, v/v) but the combined yields with the other two extreme EtOH–H$_2$O solvent mixtures (90:10 and 10:90, v/v) were 46 and 28%, respectively, thus suggesting that the possibility of conversion of malonylapiin to apiin is unlikely as one would expect accumulation of acetylapiin as

an intermediate product formed by decarboxylation of malonylapiin. However, additional extraction and stability experiments with purified malonylapiin, apiin and acetylapiin need to be performed to verify this observation. Similar HPLC profiles were observed with varying solvent polarity of MeOH and acetone solvent mixtures with water (90:10, 70:30, 50:50, 30:70 and 10:90, v/v) [Fig. 3(B) and (C)]. The major exception was that no malonylapiin was extracted with 50% MeOH. The above results indicated that extraction solvent plays a critical role in the composition of the extract and analyte recovery. To verify this observation, parsley flakes were spiked with apiin stock standard solution. Both unspiked and spiked parsley flakes were extracted with two EtOH–H$_2$O solvent mixtures (30:70 and 70:30, v/v). A seven-point calibration curve was obtained by HPLC analysis of various concentrations of apiin

stock solution ($10-150\,\mu g\,mL^{-1}$, $r^2 = 0.9997$). The extraction efficiency of standard apiin from spiked parsley flakes with EtOH–H$_2$O (30:70, v/v) was determined as 102%. However, only 89% of apiin was recovered when spiked parsley flakes were extracted with EtOH–H$_2$O (70:30, v/v). This reduction in extraction efficiency of apiin with EtOH–H$_2$O (70:30, v/v) is in agreement with the results obtained with parsley extraction without spiked apiin (Fig. 3). These results further substantiate our earlier hypothesis that retro conversion of malonylapiin to apiin did not occur in our experiments and reconfirms that solvent polarity dictates the amount and type of analyte composition recovered during extraction. Hence it is essential to evaluate systematically the extraction solvent composition, extraction procedure and conditions to optimize the recoveries of structurally diverse phenolic compounds from different food matrices as phenolics may exist as complexes with carbohydrates, proteins and other plant components.[6]

The TP content of all extracts was also estimated by the FC assay. Again, both the FC assay and HPLC analysis showed similar trends [Fig. 4(A)–(C)]. HPLC analysis (peak area) of apiin plus malonylapiin [Fig. 4(A)] and all major phenolics [Fig. 4(B)] showed similar extraction trends when the extractions were carried out with 15 different solvent compositions. As the highest combined yields of apiin plus malonylapiin and all the major identified phenolics were extracted with 50% EtOH, all comparisons with other solvent mixtures were made with that solvent mixture. Marginal differences (<10%) in combined phenolic yields were

observed when extractions were performed with 30 and 70% EtOH. However, over a 50% decrease in yield of phenolic compounds was observed with 10 and 90% EtOH [Fig. 4(A) and (B)]. Extractions with acetone–H$_2$O solvent compositions showed similar trends to EtOH–H$_2$O. Maximum combined yields of phenolic compounds were extracted with 50% acetone. Extractions with MeOH–H$_2$O solvent mixtures showed differences from extractions with EtOH–H$_2$O and acetone–H$_2$O [(Fig. 4(A)] as the highest yields of major phenolic compounds were obtained with 70% MeOH. This was due to the lower extraction efficiency of malonylapiin with 50% MeOH [Fig. 3(B)]. Estimation of TP by FC assay of all 15 extracts showed similar results to HPLC analyses [Fig. 4(C)]. Like the HPLC results, FC assay indicated maximum extraction efficiency with 50% EtOH. Marginal differences (<10%) in TP yields were observed when extractions were performed with 30:70 and 70:30 (v/v) solvent to water ratios. Lower yields of TP were observed when extractions were carried out with 10:90 and 90:10 (v/v) aqueous solvent mixtures.

Number of extraction cycles

Further investigation of the PLE method considered extraction efficiency as a function of number of extraction cycles. In this study, the extracts from each cycle were flushed into separate ASE collection vials. Four extracts were collected with each solvent mixture. Again, three solvents (EtOH, MeOH and acetone), each with five different proportions of water (90:10, 70:30, 50:50, 30:70 and 10:90, v/v), were used for extractions. All 60 extracts were analyzed by HPLC (peak area of all identified phenolic compounds) and FC assays.

The results shown in Fig. 5 depict the percentage of phenolics extracted per cycle by HPLC analysis. The percentage of phenolics extracted per cycle was calculated by dividing the sum of the HPLC peak area of all identified phenolic compounds extracted in each cycle by the sum of the HPLC peak areas of all identified phenolic compounds extracted with all four cycles. Maximum extraction of phenolic compounds per cycle (88%) was obtained with acetone–H$_2$O (50:50, v/v). Over 70% of phenolics were extracted in the first cycle, when the proportion of organic solvent to water was between 30 and 70%. In comparison, only 40–65% of phenolics were extracted in the first cycle when extractions were carried out with 10 and 90% EtOH, MeOH or acetone. The yields of the phenolic compounds extracted by the second cycle varied between 11 and 42%. The combined extraction yield of the two cycles for 15 solvent mixtures varied between 64 and 99%. Over 99% of all phenolics were extracted with three extraction cycles using 50:50 and 70:30 (v/v) EtOH, MeOH and acetone to water ratios, but the extraction efficiency varied between 84 and 98% with other solvent mixtures. TP extracted per cycle by FC assay was calculated by dividing the TP value measured per cycle by the sum of total
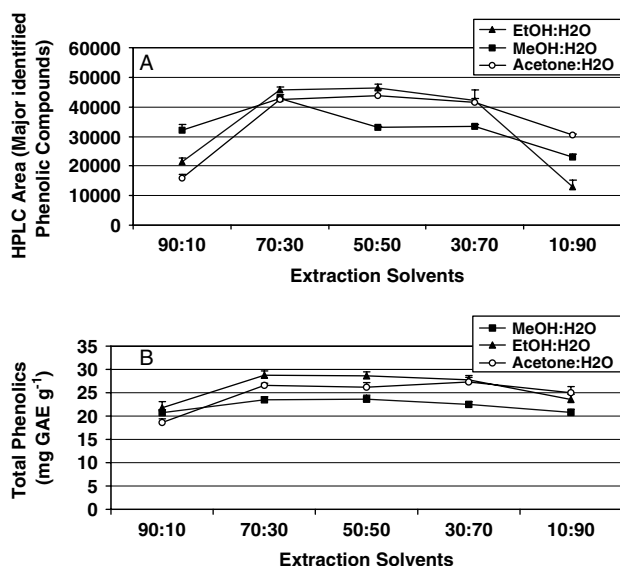

Figure 4. Influence of solvent composition on the extraction efficiency of phenolic compounds with 15 different solvent composition mixtures (solvent:H$_2$O 90:10, 70:30, 50:50, 30:70, 10:90, v/v; EtOH–H$_2$O, MeOH–H$_2$O and acetone–H$_2$O) by two different assays. HPLC analyses results with diode-array detection (350 nm) were calculated by two approaches: (A) sum of areas under of all identified major phenolic compounds; (B) total phenolics measured by Folin–Ciocalteu assay.
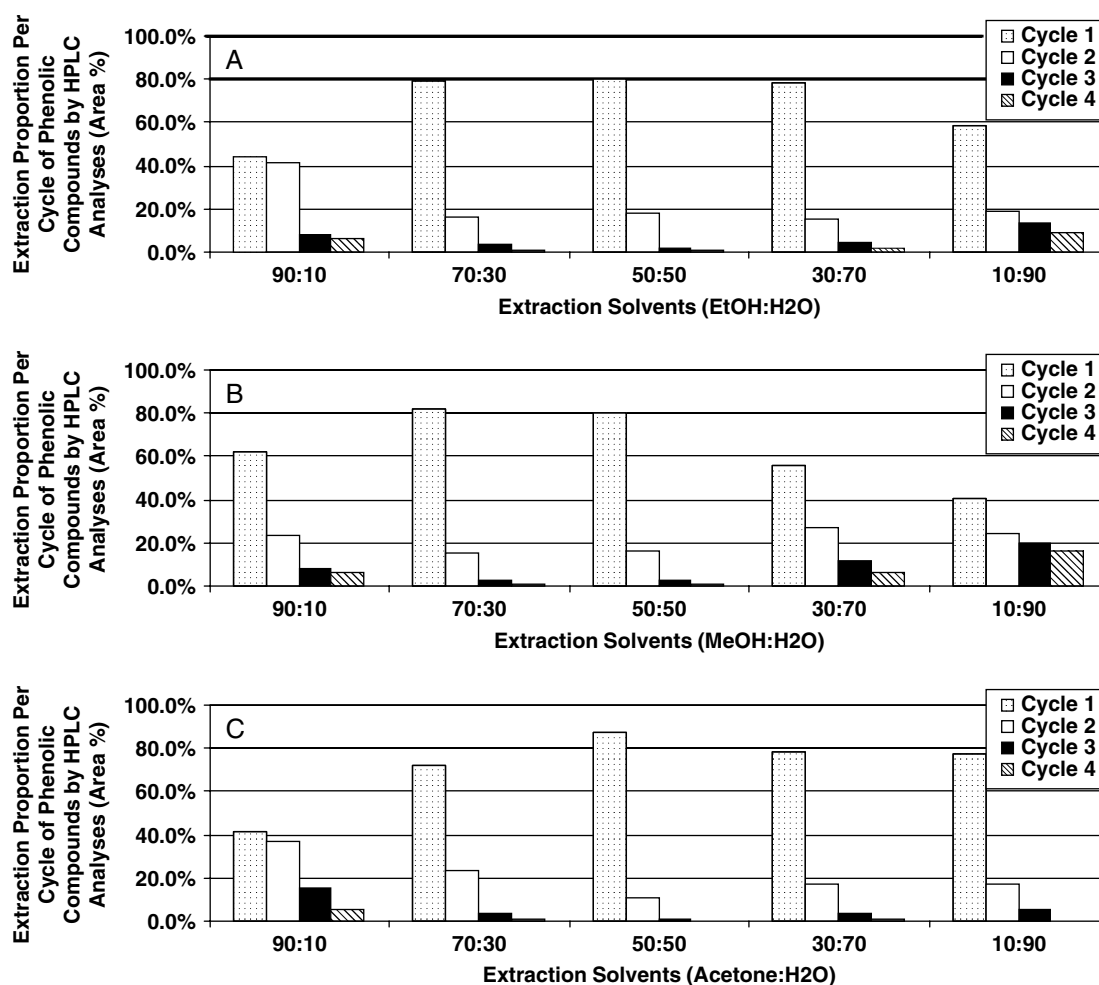
Figure 5. Influence of number of cycles on extraction of phenolic compounds from ground parsley flakes with different solvent compositions (solvent:H_2O 90:10, 70:30, 50:50, 30:70, 10:90, v/v). Samples were analyzed by HPLC with diode-array detection (350 nm). (A) EtOH–H_2O; (B) MeOH–H_2O; (C) acetone–H_2O.

TP values measured for all four cycles. The results obtained by FC assay for measurement of TP showed a similar trend. Between 63 and 93% of phenolics were extracted in the two cycles, when the ratio of organic solvent to water was between 30:70 and 70:30 (v/v). In comparison, only 34–73% of phenolic compounds were extracted in the two cycles when extractions were carried out with 10:90 and 90:10 (v/v) EtOH, MeOH or acetone to water ratios. The yields of the phenolic compounds extracted by the second cycle varied between 17 and 29%. Between 79 and 99% of all phenolics were extracted with three extraction cycles with all solvent mixtures.

The minor differences in the yield of phenolics values assayed by two independent procedures, FC assay and HPLC analysis, are attributed to differences in the assays principles. Only major phenolics that were separated and identified were quantified by HPLC, as compared with TP measured by colorimetric FC assay. In addition, FC assay, although widely used, lacks specificity. It is affected by the interferences of varying magnitude from reducing sugars, sulfites and amino acids.[18,26]

The influence of the polarity of different extraction solvent mixtures and the methodologies on the assay of phenolics from dried parsley samples was studied. The extraction efficiency of PLE was marginally better than or comparable to those of conventional extraction procedures (stirring, sonication, shaker and vortex mixing). Extractions with PLE were less time consuming and sample handling and exposure to solvent were also significantly reduced. Further optimization of various PLE parameters such as static time, flush volume, extraction time, temperature and pressure is in progress. All the parameters described in this paper in addition to the parameters currently under investigation are critical for optimization of sample preparation procedures. The amount and the composition of phenolic compounds extracted from parsley samples varied with the polarity of the extraction solvent. The present study suggested that the simple colorimetric FC assay provided results comparable to those obtained by HPLC analysis for the estimation of TP from parsley. Maximum extraction efficiency of TP was obtained when extraction were performed with 50% (v/v) ethanol solvent. The maximum amount of apiin was extracted with 30% (v/v) EtOH, MeOH or acetone. However, the highest yields of malonylapiin were obtained with 70% (v/v) EtOH, MeOH or acetone. Four cycles of

extraction were sufficient for optimum recovery of phenolic compounds from parsley flakes.

This study clearly demonstrates that it is essential to evaluate, document and optimize systematically the extraction solvent composition, extraction procedure and conditions for accurate and reproducible assay of structurally diverse phenolic compounds from different food matrices. As over 8000 different phenolics with wide ranges of polarities, forms (aglycone glycosylated, acetylated/malonylated, esterified to acids, etc.) and its association with the matrix (free or bound to cellular components) have been reported in the literature. It is impossible to develop a single, uniform sample preparation procedure for all compounds, hence optimization of sample preparation is essential for accurate analysis of different classes of phenolic compounds isolated from various matrices.

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